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MAD3 Encodes a Novel Component of the Spindle Checkpoint which Interacts with Bub3p, Cdc20p, and Mad2p

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Abstract. We show that *MAD3* encodes a novel 58-kD nuclear protein which is not essential for viability, but is an integral component of the spindle checkpoint in budding yeast. Sequence analysis reveals two regions of Mad3p that are 46 and 47% identical to sequences in the NH_2-terminal region of the budding yeast Bub1 protein kinase. Bub1p is known to bind Bub3p (Roberts et al., 1994) and we use two-hybrid assays and coimmunoprecipitation experiments to show that Mad3p can also bind to Bub3p. In addition, we find that Mad3p interacts with Mad2p and the cell cycle regulator Cdc20p. We show that the two regions of homology between Mad3p and Bub1p are crucial for these interactions and identify loss of function mutations within each domain of Mad3p. We discuss roles for Mad3p and its interactions with other spindle checkpoint proteins and with Cdc20p, the target of the checkpoint.

Key words: *MAD3* • checkpoint • *BUB3* • *CDC20* • *MAD2*

Introduction

The spindle checkpoint delays the metaphase to anaphase transition in cells with defects in the interaction between kinetochores and microtubules of the mitotic spindle (Rieder et al., 1995; for review see Rudner and Murray, 1996; Wells, 1996). This delay allows misaligned or unattached sister chromatid pairs to form a bi-polar attachment to the spindle, thereby ensuring their accurate segregation during anaphase and cytokinesis (Nicklas, 1997).

Genetic screens in budding yeast, for *mad* (mitotic arrest defective)[1] and *bub* (budding uninhibited by benzimidazole) mutants, originally identified six components of the spindle checkpoint (Hoyt et al., 1991; Li and Murray, 1991). Sequence analysis and preliminary characterization has been reported for *MAD1* (Hardwick and Murray, 1995), *MAD2* (Chen et al., 1999), *BUB1* (Roberts et al., 1994), *BUB2*, and *BUB3* (Hoyt et al., 1991). Frog and human homologues of *MAD2* and *MAD1* have conserved their checkpoint functions and localize to unattached kinetochores in tissue culture cells (Chen et al., 1996, 1998; Jin et al., 1998; Li and Benezra, 1996). *BUB1* encodes a protein kinase that binds to and phosphorylates Bub3p (Roberts et al., 1994), and mouse and fission yeast Bub1 homologues have been localized to unattached kinetochores (Bernard et al., 1998; Taylor and McKeon, 1997) in a Bub3-dependent manner (Taylor et al., 1998). In addition, it has been shown that the essential protein kinase encoded by *MPS1* also has a spindle checkpoint function (Weiss and Winey, 1996), can phosphorylate Mad1p, and that its overexpression is sufficient to activate the spindle checkpoint (Hardwick et al., 1996). It has recently been reported by a number of groups that Bub2p is likely to function on a second branch of the spindle checkpoint pathway, which is quite distinct from that in which the Mad1, Mad2, Mad3, Bub1, and Bub3 proteins function (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). Bub2p and another component of this checkpoint branch, Byr4p/Bfa1p, have both been localized to spindle pole bodies in yeast (Fraschini et al., 1999; Li, 1999).

The molecular mechanisms by which spindle defects are monitored and send a signal that induces a cell cycle delay remain poorly understood (Hardwick, 1998). In insect spermatocytes, the lack of tension on kinetochores that have only attached to microtubules from one spindle pole appears to inhibit anaphase onset (Li and Nicklas, 1995). Whether the spindle checkpoint monitors tension in somatic cells remains unclear, however, it does not regulate Mad2 binding as this checkpoint protein is only detected on one or two kinetochores in cells treated with taxol, even though none of the kinetochores in these cells are under tension (Waters et al., 1998). The role of the spindle

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[1]*Abbreviations used in this paper:* APC, anaphase-promoting complex; *BUB*, budding uninhibited by benzimidazole; *MAD*, mitotic arrest defective; ORF, open reading frame.

checkpoint proteins in unperturbed cell cycles is also uncertain, although the Mad2 protein (Gorbsky et al., 1998) and the Bub1 kinase (Taylor and McKeon, 1997) do appear to control the timing of anaphase onset in normal cell division in animal cells.

The spindle checkpoint blocks sister chromatid separation by inhibiting the anaphase-promoting complex (APC). Mad2p binds to Cdc20p, an essential activator of the APC, and in fission and budding yeasts Cdc20p mutants that cannot be inhibited by the checkpoint fail to bind to Mad2p (Hwang et al., 1998; Kim et al., 1998). In vertebrates, components of the APC have been be found in a ternary complex with Mad2p and $p55^{CDC20}$ (Fang et al., 1998; Kallio et al., 1998). Cdc20p appears to target proteins, such as Pds1p and Clb5p, for ubiquitination by the APC (Schwab et al., 1997; Visintin et al., 1997; Shirayama et al., 1999). The destruction of Pds1p is required to trigger sister chromatid separation (Cohen-Fix et al., 1996; Yamamoto et al., 1996a,b; Ciosk et al., 1998) and for mitotic exit (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999). *cdc20* mutants arrest in metaphase with high levels of Pds1p and mitotic cyclins, showing that inhibition of Cdc20p by the spindle checkpoint would be sufficient to prevent both sister chromatid separation and the destruction of the mitotic cyclins and inactivation of Cdc28p that is required for the exit from mitosis. Excess recombinant Mad2 inhibits cell cycle progression in cell-free extracts (Chen et al., 1998; Li et al., 1997) and Cdc20-mediated activation of the APC in vitro (Fang et al., 1998). The Bub2p/Byr4p-dependent branch of the spindle checkpoint has been shown to inhibit Dbf2 kinase activity (Fesquet et al., 1999), which is essential for the transition from anaphase to G1.

Here we report the cloning and characterization of budding yeast *MAD3*, whose sequence has been used to isolate homologues of Mad3 in other organisms (Taylor et al., 1998). The gene encodes a novel 58-kD nuclear protein that contains two regions of homology with Bub1p and interacts with Bub3p, Mad2p, and Cdc20p. We show that it is an integral component of the spindle checkpoint in budding yeast and discuss its possible roles in delaying anaphase onset.

Materials and Methods

Yeast Strains and Media

Table I lists the strains used in this work, all of which are derivatives of W303 except the two-hybrid strains Y 187 and Y 190, and *mps1-1* which is derived from S288c. Yeast media, growth conditions, stock solutions, and molecular techniques were as previously described (Guthrie and Fink, 1991; Hardwick and Murray, 1995). Microcolony assays were carried out as previously described (Li and Murray, 1991).

Cloning of MAD3

The *MAD3* clone was isolated from a YCP50-based genomic library (Hardwick and Murray, 1995). The *mad3-1* mutant was transformed with this library, and after 2–3 d of growth on uracil-free plates, the Ura^+ colonies were scraped off, diluted, and replated onto YPD plates containing 10 μg/ml benomyl. Plasmid DNA was prepared from benomyl-resistant colonies and individual plasmid isolates were tested for their ability to rescue *mad3-1* (KH45). One plasmid, pKH502, was able to do this and could also rescue *mad3-2* (KH160). Treatment with the Klenow fragment of DNA polymerase I to remove the overhanging bases after cutting at the unique

Table I. Yeast Strains

Strain name	Mating type	Genotype
KH 34	a	*ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 35	α	*ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 45	a	*mad3-1, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 160	a	*mad3-2, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 123	a	*mad1Δ.1::HIS3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 125	a	*mad3Δ.1::LEU2, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 173	a	*mad3Δ.2::URA3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 40	a	*cin1Δ::HIS3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 228	a	*BUB3*-$(myc)_{13}$::G418, *ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
RJ 10	a	*mad3Δ.2, BUB3*-$(myc)_{13}$::G418, *ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
RJ 11	a	*mad3-1, BUB3*-$(myc)_{13}$::G418, *ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 232	a	*mad1Δ.1::HIS3, BUB3*-$(myc)_{13}$::G418, *ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 234	a	*mad2Δ::URA3, BUB3*-$(myc)_{13}$::G418, *ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 238	a	*bub1Δ::HIS3, BUB3*-$(myc)_{13}$::G418, *ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 240	a	*bub2Δ::URA3, BUB3*-$(myc)_{13}$::G418, *ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 242	a	*mps1-1, BUB3*-$(myc)_{13}$::G418, *ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 243	a	*cdc26Δ::URA3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 244	a	*mad1Δ.1::HIS3, cdc26Δ::URA3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 246	a	*mad2Δ::TRP1, cdc26Δ::URA3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 248	a	*mad3Δ.2, cdc26Δ::URA3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 250	a	*bub1Δ::HIS3, cdc26Δ::URA3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 252	a	*bub2Δ::TRP1, cdc26Δ::URA3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 254	a	*bub3Δ::TRP1, cdc26Δ::URA3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
KH 256	a	*mps1-1, cdc26Δ::URA3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*
Y 187	α	*gal4, gal80, ura3-52, his3, leu2,3-112, his3-11, trp1-901, ade2-101 URA3::GAL-lacZ, LYS2::GAL-HIS3, SPA10::GAL-URA3*
Y 190	a	*gal4, gal80, ura3-52, his3, leu2,3-112, his3-11, trp1-901, ade2-101 URA3::GAL-lacZ, LYS2::GAL-HIS3, SPA10::GAL-URA3*

SacI site produced a clone (pKH504) that no longer rescued *mad3* mutants. To confirm that this plasmid contained the *MAD3* gene rather than a suppressor, we cloned the flanking BamHI fragment into the *URA3* integration vector pRS306 to produce pKH509. This plasmid was linearized with HpaI and transformed into wild-type haploid cells (KH35). Transformants were than mated with KH45 (*mad3-1*) and KH160 (*mad3-2*), the resulting diploid strains were sporulated and 16 tetrads dissected from each cross. In all 32 tetrads the *URA3* marker segregated away from benomyl sensitivity, demonstrating that we had cloned the *MAD3* gene.

Sequencing, Mapping, mad3 Gene Disruptions, Mutants, and Overexpression Constructs

After subcloning fragments into pBluescript, we completely sequenced a 2.2-kb segment of DNA flanking the SacI site in pKH502 using a combination of the Sequenase II DNA sequencing kit (United States Biochemical) and the ABI prism cycle sequencing kit (Perkin-Elmer). This sequence contains a 1,548-bp ORF which has since been designated YJL013c in the *Saccharomyces* genome database.

To sequence the *mad3-1* and *mad3-2* mutant alleles, genomic DNA was prepared (Ward, 1990) and the *mad3* locus amplified by PCR and then analyzed by cycle sequencing (PE Applied Biosystems). Each allele was sequenced multiple times on both strands.

Two *mad3* gene disruptions were made (see Fig. 1): one, *mad3Δ.1*, replaces the BglII-Xba1 fragment (nucleotides 702–1161, amino acids 236–388) with a BamHI-HindIII fragment containing the *LEU2* gene (pKH515). The other, *mad3Δ.2*, was made by PCR (pKH520) and replaces nucleotides 180–1441 (amino acids 60–480) with the *URA3* gene (primer 1: CCGGTACCTACAATAAAAGACGTTAAC, primer 2: GCGAATTCTTAACTTGGTTTATTTCCACC, primer 3: CGGATCCTTAGAAATGAGAGAATCA, primer 4: GATGACGCGGCCGCATAAGCGTTAATCGGACA) in pAS135.

The *MAD3* overexpression construct (pKH512) inserts a COOH-terminal myc epitope (EQKLISEEDLN) and expresses *MAD3* from the Triose Phosphate Isomerase promoter on a 2-μm, *URA3* vector (pJS209; Semenza et al., 1990). pKH513 lacks a myc tag.

To introduce the mutation into homology region I we first amplified the *MAD3* promoter using primers 3.45 (TCGAAGCTTCATATGGTTCACCAACAGCC) and 3.46 (CGCGGATCCTCTATCAAGTTAACGTCTTTT) and cloned the PCR product as a HindIII-BamHI fragment into YCPlac22 (Gietz and Sugino, 1988), producing pKH533. The HindIII site was then destroyed by filling in with the Klenow fragment of DNA polymerase I and relegating the blunt ends (pKH534). The *MAD3* ORF was then amplified in two fragments using VENT Polymerase (New England Biolabs; BamHI-NotI using primers 3.40 [CAGGATCCATGAAAGCGTACGCAAAG] and 3.48 [GGCAGCGGCCGCATTTCTTAACATATACATGAAA]) and (NotI-EcoRI using primers 3.47 [AATGCGGCCGCTGCCGAGTTAGCGTCATTTTATG] and BacM3.3 [AGGAATTCTCAACGCTGTGGTGGGTACG]) which were then cut and ligated (introducing a NotI site at their junction and replacing residues 156–159 [GIGS] with AAAA [GCGGCCGCTGCC]) into BamHI-EcoRI cut pKH534 to produce pRJ001.

Preparation of Antibodies against Mad3p, Immunoblotting, and Immunofluorescence

The *MAD3* ORF was cloned into pGEX2T (Pharmacia) as a BamHI fragment to produce pKH529. GST fusion protein expression, purification and subsequent antibody production (Berkeley Antibody Company and Diagnostics) and affinity purification was as previously described for Mad1p (Hardwick and Murray, 1995). Rabbit anti-Bub3p antibodies were prepared in a similar fashion using a pGEX-3X–expressed Bub3-GST fusion protein as antigen (construct kindly supplied by Frank Solomon, MIT), rabbit anti-Bub1p antibodies raised using a GST fusion protein containing amino acids 1–216 of Bub1p as antigen, and sheep anti-Mad2p antibodies raised against a full-length *MAD2*-GST fusion protein. Yeast extracts were made and immunoblotting performed as previously described (Hardwick and Murray, 1995). The affinity-purified anti-Mad3p, anti-Bub1p and anti-Bub3p antibodies (0.5 mg/ml, 1 mg/ml, and 1 mg/ml glycerol stocks, respectively) were used at a dilution of 1:1,000 in blotto (Harlow and Lane, 1988) and the A14 anti-myc antibody (Santa Cruz Biotechnology Inc.) at 1:2,000.

For immunofluorescence whole cells were fixed with 3.7% formaldehyde for 1 h, washed and digested for 30 min at 30°C with 50 μg/ml Zymolyase 20T (ICN) in 0.7 M sorbitol, 0.1 M KPO_4, pH 7.5, before being attached to poly-lysine–coated slides. The slides were plunged into methanol (−20°C) for 5 min and acetone (−20°C) for 30 s and then allowed to dry. Cells were blocked in blotto for 30 min, and then incubated overnight at 4°C with primary antibody diluted (anti-Mad3p at 1/2000; A14 anti-myc at 1/2,000; 9E10 at 1/500; and anti-Kar2p at 1/10,000) in blotto. Cells were washed several times in blotto and then incubated for 1 h at room temperature in FITC or Cy3-conjugated anti-rabbit or anti-rat secondary antibodies (Jackson ImmunoResearch Labs.) diluted 1:200 with blotto. Cells were washed several times with blotto and then with PBS containing 0.02% Tween 20, before mounting in Vectashield (Vector Labs.). Coverslips were sealed with clear nail polish and stored at −20°C. Images were captured with a Sensys CCD camera (Photometrics) mounted on a Zeiss Axioskop and manipulated using Quips mFISH software (Vysis).

Protein Interaction Assays

The two-hybrid fusions were constructed in the vectors pAS1-CYH2 (DNA binding domain) and pACTII (transcriptional activation domain; Clontech). All contained the full coding region, except for the Mad1 fusion which encodes amino acids 313–750 of Mad1p. The Snf4 fusion is a negative control fusion to a protein involved in the regulation of sucrose metabolism. Haploid strains containing individual fusion proteins were crossed and the resulting diploids were assayed for β-galactosidase activity. The values shown are in Miller units and are the average of three or more independent crosses.

The Mad3p two-hybrid constructs are as follows: pKH701 encodes a full-length Mad3p fusion (amino acids 1–515); pKH702 encodes amino acids 1–237; pKH703 encodes amino acids 1–409; pKH704 encodes amino acids 176–515; pKH705 encodes amino acids 308–515; pKH706 encodes amino acids 176–409; pKH707 encodes amino acids 308–409. These constructs were made by PCR amplification of the *MAD3* inserts, using either VENT polymerase or Bio-X-Act DNA polymerase (Bioline), followed by sub-cloning of the products into pAS1-CYH2, and sequencing.

Full-length *BUB3* and *CDC20* genes were cloned into pGEM3Z and expressed in rabbit reticulocyte lysates using the TNT T7 coupled transcription-translation system according to the manufacturer's instructions (Promega Corporation). For binding assays 10 μl of the translation mix was diluted with 190 μl of binding buffer (50 mM Hepes, pH 7.6, 75 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, LPC (10 μg/ml leupeptin, pepstatin, and chymostatin) and then 1 μg of GST fusion protein was added. After 30 min incubation on ice, glutathione agarose beads were added and the mix rotated at 4°C for 1 h. The beads were pelleted and washed four times in binding buffer, resuspended in sample buffer, and the bound proteins then separated by SDS-PAGE. The truncated Mad3-GST fusions contain residues 1–237 (N-term) and 176–409 (middle) of Mad3p, and the NH_2-terminal Bub1-GST fusion protein contains residues 1–216 of Bub1p. All were expressed from pGEX2T, and purified in the same manner as the full-length Mad3 fusion protein.

For coimmunoprecipitation experiments, extracts were made by bead beating as previously described (Hardwick and Murray, 1995) except that the lysis buffer was 50 mM Hepes, pH 7.6, 75 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1% Triton X-100, 1 mM PMSF, 0.5 mM DTT, and 10 μg/ml each of leupeptin, pepstatin, and chymostatin. HA-Cdc20p was immunoprecipitated with a rabbit polyclonal anti-HA antibody (Y-11; Santa Cruz Biotechnology Inc.) and immunoblotted with a rat monoclonal anti-HA antibody (3F10; Roche Molecular Biochemicals).

Results

Isolation and Sequence Analysis of MAD3

To further our molecular analysis of the spindle checkpoint in budding yeast we isolated the *MAD3* gene. The *mad3-1* mutant was transformed with a yeast genomic library and plasmids were isolated that rescued the mutant's benomyl sensitivity. One plasmid with this property also rescued the *mad3-2* allele. After restriction mapping, subcloning, and sequencing this plasmid was found to contain the open reading frame (ORF) YJL013c, which encodes Mad3p: creating a frameshift mutant in its coding se-

quence abolished its ability to complement *mad3* mutants, and genetic mapping showed that it was allelic to *mad3-1* and *mad3-2* (see Materials and Methods).

We sequenced the *mad3-1* and *mad3-2* mutant alleles and found that they both contain single point mutations. In *mad3-1* a mutation at nucleotide 1144 changes glutamate 382 to lysine, and in *mad3-2* a mutation at nucleotide 261 introduces a stop codon in place of tryptophan 87 (see Fig. 1 a).

BLAST searches with the Mad3p sequence revealed that the only protein in the budding yeast genome with significant homology to Mad3p is the previously identified spindle checkpoint protein Bub1p. Bub1p is a large 110-kD protein kinase with a COOH-terminal kinase domain, and the homology with Mad3p is towards its NH_2 terminus. Fig. 1 a indicates (in bold and underlined) the two regions of Mad3p with homology to sequences in the NH_2-terminal half of Bub1p: amino acids 64–195 of Mad3p (homology region I) are 46% identical to amino acids 44–176 of Bub1p and amino acids 343–401 of Mad3p (homology region II) are 47% identical to amino acids 304–356 of Bub1p. Fig. 1 b shows a Clustal alignment with sequences outside budding yeast and reveals that these regions of Mad3p have homology to sequences in the fission yeast and human homologues of Bub1 and also to a human protein that was identified as a Bub1/Mad3-related protein (Cahill et al., 1998; Chan et al., 1998; Taylor et al., 1998).

Figure 1. The sequence of Mad3p reveals that it has homology with Bub1p. (a) The two regions of Mad3p showing homology with Bub1p are highlighted in bold and underlined. Three mutations are indicated under the protein sequence: the *mad3-2* mutation introduces a stop codon in place of W87; the site-directed homology region I mutation replaces the GIGS motif with four alanine residues; and the *mad3-1* (homology region II) mutation replaces E382 with K. (b) Clustal alignments of homology regions I and II from related budding yeast, fission yeast, and human proteins.

MAD3 Encodes a Spindle Checkpoint Component

To confirm that Mad3p has a spindle checkpoint function we made two gene disruption constructs. One, *mad3Δ.1*, removes the COOH-terminal half of the protein by replacing amino acids 236–388 with the *LEU2* gene and the other, *mad3Δ.2*, is a more complete disruption which replaces amino acids 60–480 (82% of the *MAD3* ORF) with the *URA3* gene. Initially we tested the disrupted haploid strains using the two principal criteria for spindle checkpoint mutants: reduced ability to form colonies on benomyl-containing medium and an inability to delay cell division in response to spindle depolymerization. Fig. 2 shows that *mad3Δ.1* and *mad3Δ.2* strains have both of these phenotypes. A *cin1* strain was used as a control to show the behavior of strains containing a structural microtubule defect: the cells were benomyl sensitive, and did not divide in the microcolony assay, in which individual cells were picked onto benomyl-containing media and observed for a number of hours and their cell divisions counted. The *mad3* strains were also benomyl sensitive, and importantly they clearly continued to divide on the benomyl-containing plates (Fig. 2 b). We also carried out FACS® analyses of synchronous *mad3* and *mad3,bub2* cultures treated with nocodazole (not shown). Our results were in agreement with a number of studies of the *mad* and *bub* mutants, where it has been shown that both branches of the spindle checkpoint must be inactivated for efficient DNA rereplication to occur (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999).

In addition, previously published work has shown that, like the other *mad* and *bub* mutants, *mad3* mutants fail to maintain sister chromatid cohesion when treated with nocodazole and as a result die rapidly (Straight et al., 1996),

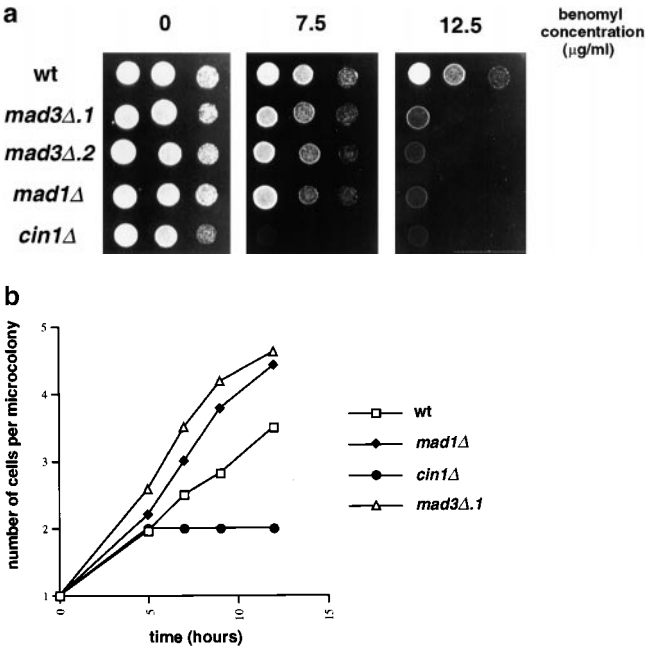


Figure 2. mad3 mutants are spindle checkpoint defective: they are benomyl sensitive and continue to divide rapidly in the presence of microtubule perturbation. (a) *mad3* deletion mutants are benomyl sensitive. Yeast cells (KH 34, KH 125, KH 173, KH 123, and KH 40) were spotted onto YPD plates containing the indicated concentrations of benomyl and photographed after 3 d growth at 23°C. (b) Microcolony analysis of the growth of individual yeast cells (strains KH 34, KH 125, KH 123 and KH 40) on YPD plates containing 12 μg/ml benomyl at 23°C. Data was collected from at least 60 cells of each genotype.

and that *mad3* mutants interact with many mitotic mutants in the same way as the previously characterized *mad1* and *mad2* mutants (Hardwick et al., 1999). Taken together, these experiments clearly demonstrate that *mad3* mutants have a very similar spindle checkpoint defect to that previously described for *mad1* (Hardwick and Murray, 1995) and *mad2* mutants (Chen et al., 1999).

To carry out biochemical analysis of Mad3p, we raised polyclonal antibodies to a bacterially expressed Mad3-GST fusion protein, and epitope-tagged Mad3p by adding a COOH-terminal myc epitope. Fig. 3 a shows that, after affinity purification, the polyclonal antibodies detected a polypeptide of 58 kD that was missing in *mad3-2* and *mad3Δ* mutants and was still present in the *mad3-1* strain. This immunoblot is consistent with our sequence analysis of the *mad3* alleles and shows that our polyclonal antibodies are specific for Mad3p.

We used this antibody to analyze the abundance of Mad3p through the cell cycle (Fig. 3 c): wild-type yeast cells were synchronized in G1 with alpha-factor and then washed and released into rich growth media, either with or without the addition of nocodazole. Samples were taken every 20 min and analyzed by immunoblotting with anti-Mad3p, anti-Mad1p, and anti-Clb2p (a mitotic cyclin) antibodies. It is clear from this experiment that unlike Clb2p, which is absent in G1 and high in mitosis, the abundance of Mad3p does not alter during the cell cycle. In addition, unlike Mad1p which becomes hyperphosphorylated upon nocodazole treatment (Hardwick and Murray, 1995), there was no obvious posttranslational modification of Mad3p that could be resolved by SDS-PAGE, either during the cell cycle or upon checkpoint activation.

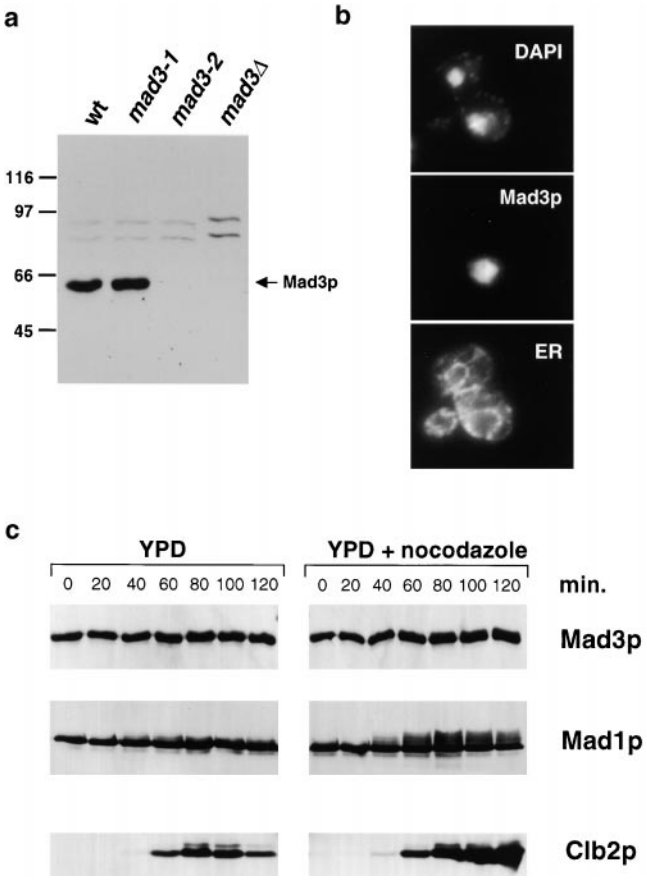


Figure 3. Antibodies to Mad3p reveal that it is a nuclear protein, and that its abundance is not cell cycle regulated. (a) Immunoblots of yeast extracts with anti-Mad3p antibodies. Whole cell extracts made from wild-type (KH 34), *mad3Δ.2* (KH 173), *mad3-1* (KH 45), and *mad3-2* (KH 160) strains were immunoblotted with affinity-purified anti-Mad3p antibodies. A strong signal at ~58 kD is seen in the wild-type and *mad3-1* extracts, which is missing in the *mad3Δ* and *mad3-2* strains indicating that it is specific for Mad3p. (b) Immunofluorescence staining of yeast with anti-Mad3p antibodies. An asynchronous culture of wild-type cells containing myc-tagged Mad3p expressed from a multi-copy plasmid were stained with DAPI, affinity-purified anti-Mad3p antibodies, and anti-Kar2p as a marker for the nuclear envelope and endoplasmic reticulum. Mad3p levels are very variable due to its expression from a multi-copy vector, but in all cases is confined to the nucleus, as delineated by the Kar2p signal in the nuclear envelope. Similar results were obtained with an anti-myc antibody. (c) Mad3p levels are not cell cycle regulated. Wild-type cells (KH 34) were arrested with the mating pheromone alpha-factor and then released into YPD with and without the addition of 15 μg/ml nocodazole. Samples were taken every 20 min for a period of 2 h, whole cell extracts were prepared, and then immunoblotted with affinity-purified antibodies specific for Mad3p, Mad1p, and the mitotic cyclin Clb2p. Unlike Mad1p, which becomes hyperphosphorylated upon nocodazole treatment, and Clb2p, which is regulated at the level of abundance, Mad3p levels are constant and the protein displays no obvious signs of modification.

Our immunofluorescence analysis has failed to detect wild-type levels of Mad3p. However, strains containing a multi-copy vector expressing Mad3p-myc from the TPI promoter (pKH512) show a general nuclear localization for Mad3p when stained with either anti-myc or anti-Mad3p antibodies (Fig. 3 b). As this plasmid has a 2-μm origin of replication there are widely differing levels of expression in the population, due to the wide variation in plasmid copy number. However, nuclear staining was observed at all detectable levels of expression suggesting that the protein is likely to be nuclear in wild-type cells. In some cells expressing high levels of Mad3p the antibodies clearly labeled more of the nucleus than was DAPI-stained. To confirm that the Mad3p staining was entirely nuclear we performed double label immunofluorescence experiments using anti-tubulin (not shown) and anti-Kar2p antibodies. The latter is a soluble protein of the endoplasmic reticulum and gave clear staining of the nuclear envelope, within which Mad3p was restricted (Fig. 3 b).

Mad3p Binds to Bub3p and Cdc20p

Mad3p has two regions of homology with Bub1p, a protein that has been shown to bind to Bub3p (Roberts et al., 1994). Therefore, we wanted to test whether Mad3p could also interact with Bub3p. Initially, we used the two-hybrid assay to test whether Mad3p interacts with any of the known checkpoint components. Fig. 4 a shows that full-length Mad3p interacts with both Bub3p and Cdc20p in the two-hybrid assay, but not significantly with Mad1p, Mad2p, Bub1p, or Bub2p. To determine which portion of Mad3p contains the binding sites for Bub3p and Cdc20p, a number of Mad3 deletion constructs were tested. Fig. 4 b shows that homology region I of Mad3p, which contains the longer region of homology to Bub1p, is not necessary for its interaction with Bub3p, and that homology region II is necessary and sufficient for Bub3p binding. Conversely, Cdc20p was found to interact with fragments containing homology region I of Mad3p. Bub1p also contains a homologous region I and we next tested whether it could also interact with Cdc20p. In the two-hybrid assay homology region I of Bub1p does interact with Cdc20p: an average β-galactosidase activity of 100 Miller units was measured, comparing favorably with its Bub3p interaction of 55 Miller units.

To confirm the above interactions between Mad3p, Bub3p, and Cdc20p we carried out a number of coimmunoprecipitation experiments. Two *mad3* mutants were analyzed: *mad3-1* contains a point mutation in homology region II (E382K), and we made a site-directed mutant in homology region I (changing the sequence $GIGS_{159}$ to AAAA). In these experiments, all checkpoint proteins were expressed at wild-type levels; in some Bub3p was tagged with 13 copies of the Myc epitope (Bub3-Myc13), and Cdc20p was tagged with three copies of the HA epitope (Cdc20-HA3). Fig. 5 a shows that a Bub3p immunoprecipitate contained wild-type Mad3p, and that the homology region I mutation had no effect on this binding, but that the single amino acid change in the homology region II mutant was sufficient to abolish Bub3p binding. Fig. 5 b shows that the homology region I mutation, but not the homology region II mutation, abolished the

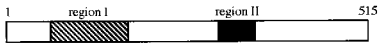
a

ACTIVATION DOMAIN FUSION

BINDING DOMAIN FUSION	Mad1	Mad2	Mad3	Bub1	Bub2	Bub3	Cdc20	Snf4
Mad3	0.7	0.9	5.4	0.3	0.3	136.4	76.0	0.2

	Cdc20	Bub3	Snf4
Bub1	100.0	55.0	0.3

b



MAD3 2-Hybrid Fusions	Bub3p	Cdc20p
pKH701	136.4	76.0
pKH702 (237)	0.4	45.2
pKH703 (409)	126.8	48.0
pKH704 (176)	77.6	0.5
pKH705 (308)	76.9	1.1
pKH706 (176–409)	113.1	0.9
pKH707 (308–409)	185.5	3.5

Figure 4. Mad3p and Bub1p both have two-hybrid interactions with Bub3p and the target of the spindle checkpoint, Cdc20p. (a) A haploid strain containing a fusion between the Gal4 DNA binding domain and Mad3p or Bub1p was crossed with strains containing fusions between the transcriptional activation domain and the indicated checkpoint proteins or Snf4p. β-Galactoside activity of the resulting diploids is shown in Miller units and is the average of at least three independent crosses. (b) Mapping the Bub3p and Cdc20p interaction domains of Mad3p. A series of Mad3p truncations was tested for Bub3p and Cdc20p interaction in the two-hybrid assay, identifying two distinct interaction domains.

Cdc20p interaction. These immunoprecipitation results are entirely consistent with the two-hybrid data and also show that neither of the mutations disrupts the entire structure of the protein. When taken together, they show that homology region I of Mad3p is needed to bind to Cdc20p, and that homology region II of Mad3p is necessary for Bub3p binding. What is the in vivo phenotype of these *mad3* mutations? Fig. 5 c shows that both the homology region I and the homology region II mutation lead to a benomyl-sensitive phenotype. Strains containing the region I mutation were as benomyl sensitive as a *mad3* null mutant, and the region II mutation was almost as severe. This indicates that both the Bub3p and the Cdc20p interaction are important for Mad3p checkpoint function.

To test whether either region of homology was sufficient for the Bub3p or Cdc20p interaction, we performed in vitro binding experiments using bacterially expressed Mad3-GST fusion proteins. The purified fusion proteins were incubated with reticulocyte lysates within which either Bub3p or Cdc20p had been translated and radiolabeled, and then pulled down with glutathione agarose beads. Fig. 5 d shows that radiolabeled Bub3p bound efficiently to the Mad3-GST fusion and that amino acids 176–409 of Mad3p (containing homology region II) were suffi-

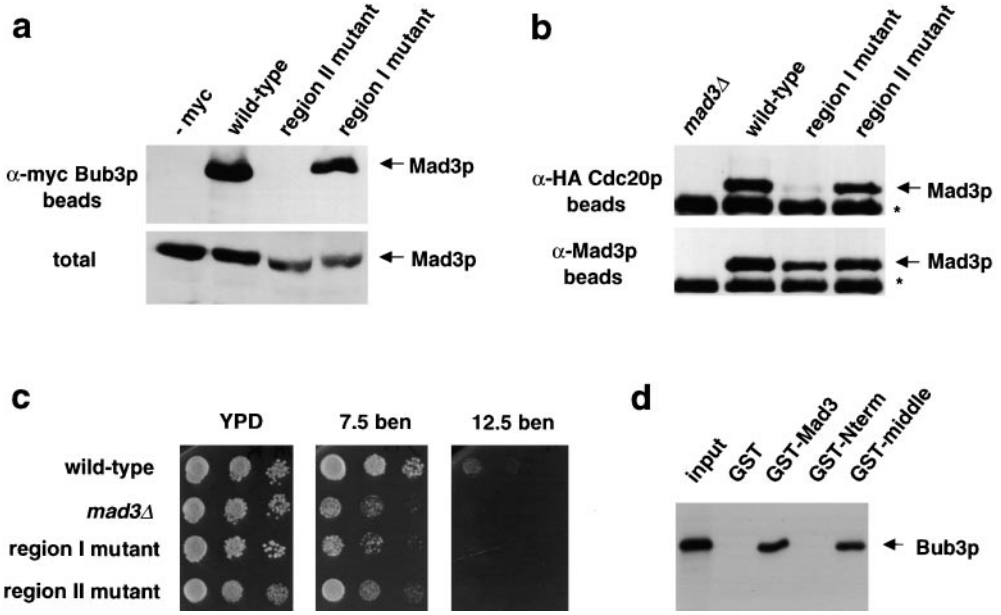


Figure 5. Functional analysis of the two homology regions of Mad3p confirms that they form two distinct interaction domains and shows that both are required for checkpoint function. (a) Wild-type, but not homology region II mutant, Mad3p was coimmunoprecipitated with Bub3p. Extracts were made from four yeast strains: a control strain lacking a myc tag (KH 34); a wild-type strain containing myc-tagged Bub3p (KH 228); a *mad3Δ* strain containing the plasmid pRJ001 (encoding the *mad3* homology region I mutant $GIGS_{159}$ > AAAA) and myc-tagged Bub3p (RJ 10); and a *mad3* homology region II mutant (*mad3-1*) containing myc-tagged Bub3p (RJ 11). Bub3p was immunoprecipitated from these extracts and the lysates and immunoprecipitates were then immunoblotted with anti-Mad3p antibodies. (b) Wild-type, but not homology region I mutant, Mad3p was coimmunoprecipitated with Cdc20p. Extracts were made from four yeast strains: a *mad3Δ* strain (a Ura^- derivative of KH173); a wild-type strain (KH 34); a *mad3Δ* strain containing the plasmid pRJ001 (encoding the *mad3* homology region I mutant); and a *mad3* homology region II mutant (KH 45). All four strains contained pLH68 which encodes HA-tagged Cdc20p. Mad3p and Cdc20p were immunoprecipitated from these extracts and the immunoprecipitates were then immunoblotted with anti-Mad3p antibodies. *Immunoglobulin heavy chains from the immunoprecipitation. (c) Benomyl sensitivity of homology region I and region II *mad3* mutants. Cells were spotted out on YPD plates and plates with 7.5 and 12.5 μg/ml benomyl and then photographed after 3 d growth at 24°C. (d) The central portion of Mad3p is sufficient for Bub3p binding. Mad3-GST fusions (Nterm contains amino acids 1–237 and middle contains 176–409) were mixed with reticulocyte lysates containing radiolabeled Bub3p, and the GST fusions were pulled down with glutathione agarose beads to determine whether they had bound Bub3p.

cient for this interaction. Unfortunately, Cdc20p bound at significant levels to the GST control protein preventing a reliable assessment of whether homology region I of Mad3p is sufficient for binding to Cdc20p

Cell Cycle Regulation of Mad3p Complexes

To determine whether either the Mad3p–Bub3p or the Mad3p–Cdc20p complexes were cell cycle regulated we performed coimmunoprecipitation analyses in extracts made from cells arrested in alpha factor, hydroxyurea and nocodazole. Fig. 6 a shows that there was no difference in the amount of Bub3p associated with Mad3p at different points in the cell cycle. There was an increased amount of Cdc20p associated with Mad3p in the later stages of the cell cycle, however, this may simply reflect the increased abundance of Cdc20p in those lysates (data not shown). Fig. 6 a also reveals that there was an increased amount of Mad2p associated with Mad3p in mitosis, and that there was no association with Mad1p.

Dependency of Mad3p Complexes on Other Checkpoint Components

We have previously shown that formation of a Mad1p–Mad2p complex is not dependent on the presence of the other checkpoint proteins (Chen et al., 1999). To determine whether the Mad3p complexes were dependent on other checkpoint components, we attempted to coimmunoprecipitate them from yeast strains specifically lacking a checkpoint component. To analyze the Mad3p–Bub3p association, we immunoprecipitated Mad3p from checkpoint mutant strains containing myc-tagged Bub3p. Immunoblotting these precipitates with anti-myc antibodies (Fig. 6 b) revealed that none of the known checkpoint proteins are required for the Mad3p–Bub3p interaction.

To analyze the Mad3p–Cdc20p and Mad3p–Mad2p interactions, we wished to perform a similar experiment, and for these interactions it was important to ensure that all strains arrested in mitosis. As *mad* and *bub* strains do not arrest well in nocodazole, we introduced a temperature-sensitive APC mutation (*cdc26Δ*) into the checkpoint mutants. When shifted to 37°C, such strains arrest in metaphase due to an inability to degrade the anaphase inhibitor Pds1p (Hwang and Murray, 1997). These strains were grown to log phase and treated with nocodazole for 3 h at 37°C. Mad3p and Cdc20-HAp were then immunoprecipitated from native extracts. Fig. 7 shows the results of immunoblotting such immunoprecipitates for Mad3p, Cdc20-HAp, and Mad2p. Fig. 7 a reveals that Cdc20-HAp was present in Mad3p immunoprecipitates at wild-type levels in *bub2* extracts, but at reduced levels or was entirely absent in *mad1*, *mad2*, *bub1*, *bub3*, and *mps1* extracts. Anti-Mad2p immunoblots revealed that a Mad3p–Mad2p complex was only detectable in wild-type and *bub2*

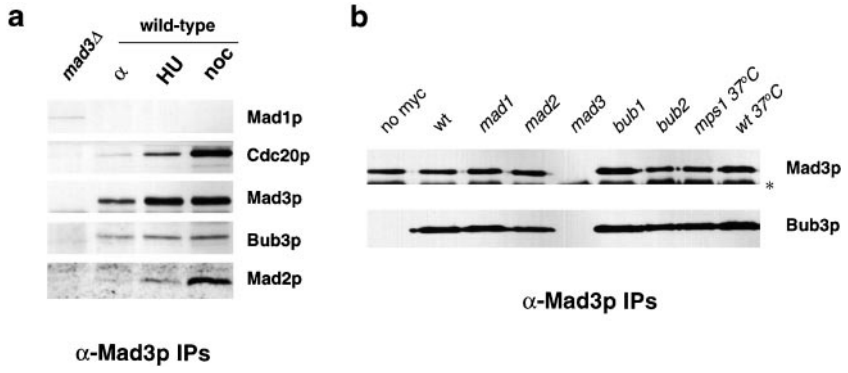


Figure 6. Cell cycle regulation of Mad3p complexes and the lack of dependency of Mad3p–Bub3p complex formation on other checkpoint proteins. (a) Levels of Mad3p–Cdc20p and Mad3p–Mad2p interaction vary through the cell cycle, but the level of Mad3p–Bub3p complex remains constant. Wild-type yeast (KH 34) was grown to log phase and then arrested in G1 (with the mating pheromone alpha factor), in S phase (with hydroxyurea) or in mitosis (with nocodazole). Lysates were prepared from these cells, and from a control strain lacking Mad3p (KH 173), and Mad3p was immunoprecipitated using affinity-purified antibodies. The immunoprecipitates were then separated by SDS-PAGE and immunoblotted with antibodies specific for Mad1p, Cdc20p (anti-HA), Mad3p, Bub3p, and Mad2p. (b) Mad3p–Bub3p complex formation does not require the other known checkpoint proteins. Strains KH 232–242, all of which contain myc-tagged Bub3p, were grown to log phase, whole cell extracts were prepared, and Mad3p was immunoprecipitated. The immunoprecipitates were then separated by SDS-PAGE and immunoblotted with antibodies specific for Bub3p (anti-Myc) and Mad3p. *Immunoglobulin heavy chains from the immunoprecipitation.

mutant strains. This is consistent with recent work showing that Bub2p lies on a quite different branch of the spindle checkpoint to the other Mad and Bub proteins (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). Fig. 7 b shows that the lack of a Mad3p–Mad2p interaction does not simply reflect the lack of a Cdc20p–Mad2p complex in the mutant extracts, as Mad2p could be detected in Cdc20p immunoprecipitates made from the same extracts. Wild-type levels of Mad2p–Cdc20p complex were detected in *mad3* and *bub2* strains, confirming that Mad3p is not required for the formation of this complex (Hwang et al., 1998). The levels of Mad2p–Cdc20p were clearly reduced in the other checkpoint mutants. Mad1p could not be detected in either the Mad3p or the Cdc20p immunoprecipitates (not shown), showing that while it may aid in their formation it does not form a stable component of such complexes.

These experiments show that Mad3p was not required for Mad2p–Cdc20p complex formation, in fact in some experiments the Mad2p–Cdc20p complex was found at slightly higher levels in *mad3* than in wild-type extracts. However, in both wild-type and *bub2* strains, where nocodazole treatment would lead to an inhibition of Cdc20p-dependent APC activity, Mad3p was stably associated with both Cdc20p and Mad2p. We propose that it is this association of Mad3p with both Mad2p and Cdc20p that is crucial for inhibition of the APC and allows Mad3p to play its role in the spindle checkpoint.

Discussion

We have shown that *MAD3* encodes a novel 58-kD com-

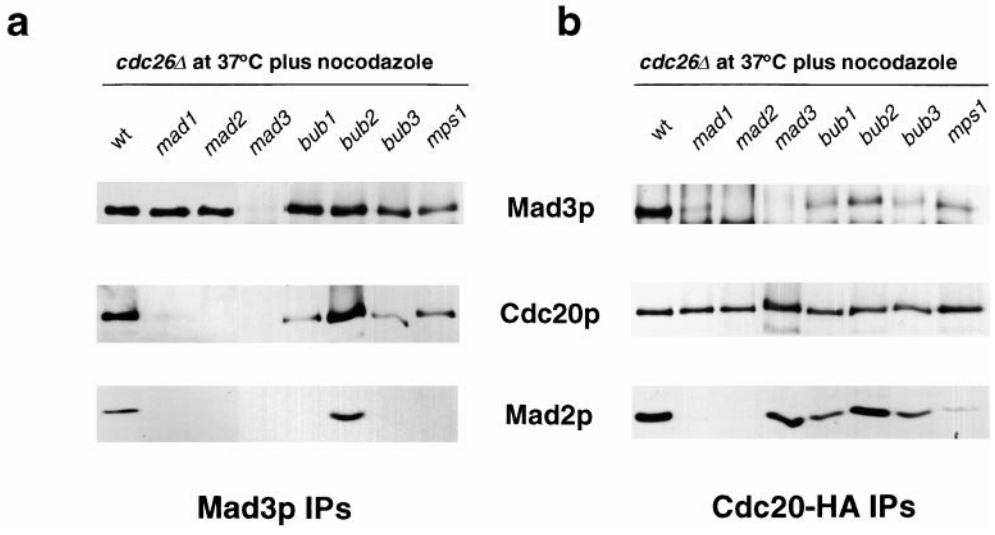


Figure 7. Dependence of Mad3p–Cdc20p, Mad3p–Mad2p, and Cdc20p–Mad2p complex formation on the other checkpoint proteins. Strains KH 243–256, all of which contain the temperature-sensitive *cdc26Δ* and pLH68L2 (*CDC20*-HA), were grown to log phase and then nocodazole was added to their growth media for 3 h at 37°C. Whole cell extracts were prepared and split into two aliquots from which Mad3p and Cdc20p were immunoprecipitated. The immunoprecipitates were then separated by SDS-PAGE and immunoblotted with antibodies specific for Cdc20p (anti-HA), Mad3p and Mad2p. (a) Mad3p immunoprecipitations reveal that the Mad3p–Cdc20p complex was low in all checkpoint mutants, except for *bub2*, and was entirely absent in *mad2*. A Mad3p–Mad2p association could only be detected in wild-type and *bub2* strains. (b) Cdc20p immunoprecipitations reveal that wild-type levels of Mad2p–Cdc20p were present in *mad3* and *bub2* extracts. The Mad2p–Cdc20p levels were reduced in all other mutants.

ponent of the spindle checkpoint that has significant homology to the NH_2-terminal region of the Bub1 protein kinase. Gene disruption experiments revealed that lack of Mad3p abolishes spindle checkpoint function, and mutational analyses indicate that two regions of Mad3/Bub1 homology are critical for Mad3p's function. A number of approaches have been taken to show that homology region I of Mad3p is required for its interaction with Cdc20p, and that homology region II defines a Bub3p binding site.

Checkpoint Function of Mad3p

We used a number of assays to show that *mad3* strains are spindle checkpoint defective. *Mad3* strains show a similar benomyl sensitivity to *mad1* and *mad2* mutants. More importantly, in microcolony assays they show the same behavior as *mad1* and *mad2* and continue to divide in the presence of microtubule perturbations. This is quite unlike the behavior of wild-type cells or strains with structural microtubule defects, such as *cin1* or *tub* mutants, which arrest in mitosis in response to microtubule depolymerization. Mutational analysis showed both homology regions to be important for checkpoint function. The region I mutant, which failed to bind to either Cdc20p or Mad2p (data not shown), behaved as a null mutant in benomyl sensitivity and microcolony assays. The region II mutant, which failed to bind Bub3p, is somewhat less benomyl sensitive. The reason for this is unclear and is currently under investigation.

Our immunofluorescence analysis revealed that Mad3p is nuclear in yeast, but the protein can only be detected when overexpressed. The only components of the budding yeast spindle checkpoint that have been localized at their wild-type expression level are Mad1p, Bub2p and Byr4p. Mad1p is found in a punctate nuclear pattern (Hardwick and Murray, 1995), and Bub2p and Byr4/Bfa1p are seen at spindle poles (Fraschini et al., 1999; Li, 1999). When they are overexpressed, Mad2p is found throughout the cell (Chen, R.-H., personal communication) and Bub1p and Bub3p are both nuclear proteins (Roberts et al., 1994). In vertebrate cells, homologues of Mad1, Mad2, Mad3 (Bub1R), Bub1, and Bub3, have all been shown to localize to kinetochores that have not captured microtubules (Chen et al., 1996, 1998; Li and Benezra, 1996; Taylor and McKeon, 1997; Jin et al., 1998; Taylor et al., 1998) and the Mad1, Mad2, Mad3, and Bub1 homologues have been shown to play a role in the spindle checkpoint (Chen et al., 1996, 1998; Li and Benezra, 1996; Taylor and McKeon, 1997; Chan et al., 1999). These observations suggest that many of the budding yeast checkpoint proteins, including Mad3p, are also likely to localize to the kinetochore.

Mad3p Interacts with Other Checkpoint Components

Bub1p and Bub3p form a complex in budding yeast, and amino acids 141–609 of Bub1p are sufficient for that interaction (Roberts et al., 1994). The homology between Mad3p and Bub1p prompted us to ask whether Mad3p could also bind to Bub3p. A combination of coimmunoprecipitation, two-hybrid assays, and in vitro binding experiments showed that Mad3p does bind to Bub3p. We found that homology region I was not necessary for Bub3p binding and that a 95–amino acid Mad3p segment containing homology region II was sufficient for this interaction in the two-hybrid assay. Sequencing of the *mad3-1* allele revealed that it contains a single point mutation (E382K) within homology region II, and coimmunoprecipitation analyses show that this abolishes Bub3p binding. In vitro binding experiments showed that amino acids 176–409 of Mad3p (which lacks homology region I) were sufficient for Bub3p binding. Our results are in agreement with vertebrate experiments showing that homology region II of the human Bub1 and Mad3/Bub1-related proteins is required for their interaction with Bub3, and for their localization to the kinetochore (Taylor et al., 1998), suggesting that this region targets the recruitment of these proteins to kinetochores that lack bound microtubules.

We have shown by coimmunoprecipitation that the Mad3p–Bub3p interaction is not cell cycle regulated and does not require the presence of the other known checkpoint proteins. We have obtained similar results for the Bub1p–Bub3p interaction (Brady, D.M., and K.G. Hardwick, manuscript submitted for publication), and we have previously reported such behavior for the Mad1p–Mad2p complex in budding yeast (Chen et al., 1999). Thus, there are a number of spindle checkpoint protein complexes that are formed constitutively. The region II mutation (*mad3-1*), and several *mad1* mutations (Chen et al., 1999) show that formation of these complexes is required for checkpoint function. This could be because it is only as a part of a complex that certain checkpoint proteins are recruited to kinetochores (see below).

We have also shown that Mad3p can interact with Cdc20p which is the target of the branch of the spindle checkpoint that monitors kinetochore behavior. Two-hybrid analysis revealed a strong interaction between Mad3p and Cdc20p and also between Bub1p and Cdc20p. Deletion analysis suggested that the NH_2-terminal two-thirds of Mad3p, which contains homology region I, was required for this interaction. To test the importance of homology region I, we mutated conserved residues within it ($GIGS_{159} > AAAA$) and constructed a yeast strain containing only this mutant form of Mad3p. Coimmunoprecipitation analysis showed that the mutant Mad3p failed to bind well to Cdc20p and to Mad2p (data not shown), but that it still bound Bub3p. The resulting *mad3* strains were benomyl sensitive indicating that the Mad3p–Cdc20p/Mad2p interaction is essential for its checkpoint function.

Stable complex formation between Mad3p and Cdc20p was previously shown to be dependent on the presence of the Mad1 and Mad2 proteins in yeast extracts (Hwang et al., 1998), which have themselves been shown to form a tight complex (Chen et al., 1999). We confirmed this result, although in certain experiments we found very low but detectable levels of the Mad3p–Cdc20p and the Mad2p–Cdc20p complex in nocodazole-treated *mad1* mutant extracts. The Mad3p–Cdc20p complex was also found at low levels in *bub1* and *bub3* null strains and in *mps1* ts strains at their restrictive temperature, but was never detected in the *mad2* mutant. From this we conclude that Mad2p function is essential for a stable Mad3p–Cdc20p interaction, probably because it is itself part of the complex (see model in Fig. 8). The low levels of Mad3p–Cdc20p in the other *mad/bub/mps1* strains could reflect a reduced stability of

the complex in the absence of other checkpoint components, or that those proteins also play some role in its formation. Note, it was not due to a failure to arrest in mitosis upon nocodazole treatment, as our strains contained a *cdc26* deletion and the experiment was carried out at its restrictive temperature ensuring a metaphase arrest. We suggest that some of the checkpoint proteins act primarily to recruit other members of the checkpoint to the kinetochore where high local concentrations stimulate reactions amongst them. The best candidates for this recruiting function are Mad1p, which has been shown to recruit Mad2p to kinetochores in *Xenopus* (Chen et al., 1998), and Bub3p which may recruit both Bub1p and Mad3p to kinetochores (Taylor et al., 1998). Bub2p does not appear to have a role to play in the formation of the Mad3p–Cdc20p/Mad2p complex, which is in agreement with its proposed role on a separate branch of the checkpoint pathway.

Although we saw Mad2p associated with both Mad3p and Cdc20p, Mad1p does not appear to be a stable component of such complexes, as it was not detectable in either the Mad3p or the Cdc20p immunoprecipitation (Fig. 6 a and data not shown). This is despite our previous observation that Mad1p and Mad2p form a very tight complex (Chen et al., 1999). These results suggest that Mad2p is present in at least two complexes: an association with Mad1p that is required to recruit Mad2p to kinetochores, and another association with Cdc20p and Mad3p. The Mad3p–Cdc20p/Mad2p complex does not require nocodazole treatment for its formation, as it is formed in *cdc26Δ* strains arrested in metaphase at their restrictive temperature (data not shown). However, we propose that the Mad3p–Cdc20p/Mad2p complex is the one through which Mad3p exerts its crucial role in inhibiting the metaphase-anaphase transition. This idea is supported by the observation that we were only able to detect significant levels of Mad3p in association with both Cdc20p and Mad2p in extracts from wild-type and *bub2* cells, and they are the only cells in which a checkpoint arrest would be maintained.

Further in vitro binding experiments using recombinant proteins will be needed before the formation and interactions of the above checkpoint protein complexes can be fully understood. It has been argued from in vitro studies that tetrameric Mad2p when complexed with Cdc20p inhibits activation of the APC (Fang et al., 1998), and it will therefore be of particular interest to test the effect of Mad3p on the ability of Mad2p to inhibit Cdc20p function and thereby APC activity. It has recently been reported that hBubR1 binds the APC in mitotic cells (Chan et al., 1999). We have tested whether Mad3p or Bub1p can interact with a component of the APC (Cdc23p), or with the Cdc20p-related protein Hct1p, but have found no evidence for such complexes by immunoprecipitation (Hardwick, K.G., data not shown).

It is unclear why homology region I is so well conserved between Mad3p and Bub1p. Our two-hybrid experiments suggested that Bub1p also binds Cdc20p, however, we have struggled to detect a Bub1p–Cdc20p complex by coimmunoprecipitation from wild-type cells (not shown). We have detected a Bub1p–Cdc20p complex in *mad* mutant extracts, where there is little if any Mad3p–Cdc20p, suggesting that Mad3p normally outcompetes Bub1p for

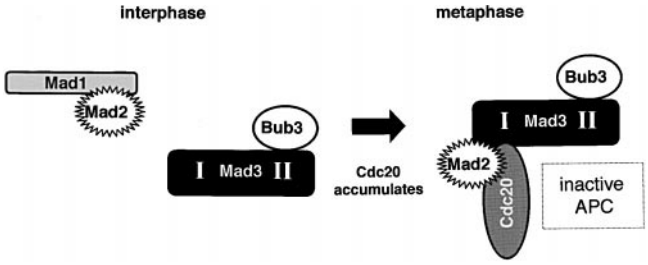


Figure 8. A model describing Mad3p interactions. The two regions of homology between Mad3p and Bub1p are labeled I and II. Mad3p associates with Bub3p throughout the cell cycle. Once Cdc20p accumulates, Mad3p can also be found in association with Cdc20p and Mad2p. The formation or stability of this complex is affected by mad1, bub1 and mps1 mutations, but the roles of those proteins remain unclear (see text for details).

interaction with Cdc20p. Further experiments have revealed that Bub1p forms a stable association with Mad1p in cells in which the spindle checkpoint has been activated (Brady, D.M., and K.G. Hardwick, manuscript submitted for publication). As there is no Mad3p or Cdc20p associated with the putative Mad1p–Bub1p/Bub3p complex we have proposed that it has a signaling function, acting upstream of Mad3p–Cdc20p/Mad2p.

Mad/Bub Proteins and Kinetochore Signaling

The components of the spindle checkpoint may function as large multi-protein complexes. Previous work has shown that Mad3p and Mad2p can be coimmunoprecipitated with Cdc20p (Hwang et al., 1998), that Mad2p and Mad1p form a tight complex (Chen et al., 1999), and that Bub1p can be coimmunoprecipitated with Bub3p (Roberts et al., 1994). Here we have confirmed the Mad3p–Cdc20p interaction and shown that Mad3p also interacts with Bub3p and Mad2p. Thus, six checkpoint components and a target of the spindle checkpoint have been shown to interact physically, suggesting that much of the checkpoint apparatus functions as one or more large multi-protein complexes.

Vertebrate homologues of Mad1, Mad2, Mad3, Bub1, and Bub3 bind to all kinetochores in cells that have been arrested in mitosis by microtubule polymerization inhibitors, and specifically localize to microtubule-free kinetochores during spindle assembly in normal cells (Chen et al., 1996, 1998; Li and Benezra, 1996; Taylor and McKeon, 1997; Chan et al., 1998, 1999; Jin et al., 1998; Taylor et al., 1998). The combination of the vertebrate and yeast results suggest a plausible pathway for the spindle checkpoint: microtubule-free kinetochores attract recruiting proteins, such as Mad1p and Bub3p and these in turn recruit other proteins (Mad2p [Chen et al., 1998], Mad3p, and Bub1p [Taylor et al., 1998]) which bind to and inhibit Cdc20p, thus preventing sister chromatids from separating. This scheme leaves several important questions: how do checkpoint components distinguish between kinetochores with and without bound microtubules, how does interaction of checkpoint components with the kinetochores lead to the inhibition of Cdc20p, how can a single microtubule or tension-free kinetochore inactivate the majority of the

Cdc20p in the cell, and do the other identified components of the checkpoint (Mps1p and Cdc55p) also participate in a kinetochore-bound signaling machine?

A physical link between spindle checkpoint proteins and a kinetochore-bound motor protein was recently uncovered through the analysis of hBubR1 (Chan et al., 1998, 1999), which we believe to be the human homologue of Mad3p. The kinesin motor, CENP-E was found to interact with hBubR1, both in a two-hybrid screen and by coimmunoprecipitation. In addition, these proteins colocalized at kinetochores, particularly those that had yet to align at the metaphase plate. The hBubR1 observations and our yeast biochemical studies suggest that Mad3p could also have a role to play in the recruitment of Cdc20p/Mad2p to kinetochores.

Recent results suggest that lesions in the spindle checkpoint play an important role in human cancer. Four cell lines derived from human colorectal cancers were found to carry mutations in the human homologue of Bub1 or the Bub1/Mad3-related gene (Cahill et al., 1998). These observations suggest that mutational inactivation of spindle checkpoint components is directly related to the chromosomal instability associated with colorectal and other cancers. The human Bub1/Mad3-related protein differs from budding yeast Mad3p by containing a COOH-terminal protein kinase domain. Although this feature gives it a similar overall structure to Bub1p, the protein kinase domain of the human Bub1/Mad3-related protein is clearly different to that of human and budding yeast Bub1 (Taylor et al., 1998). One explanation of these features is that the *BUB1* and *MAD3* genes are the product of an ancient gene duplication and that the protein kinase domain of yeast Mad3p has either been lost during evolution or separated into a different polypeptide. We have recently identified a fission yeast Mad3 homologue (Hardwick, K.G., and D. Millband, unpublished data) and found that as in budding yeast it lacks a protein kinase domain, but resolving this issue will require determining the structures of the *MAD3/BUB1* related genes in other organisms.

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